Osmotic Induction of Stress-Responsive Gene Expression in the Lobster *Homarus americanus*

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Abstract. The American lobster, *Homarus americanus*, encounters osmotic stress throughout its life cycle. To understand the molecular basis of osmotic stress responses *in vivo*, we used homologous cDNA probes to characterize the mRNA patterns of lobster HSP70 (=70-kDa heat-shock protein), HSP90 (=90-kDa heat-shock protein), and polyubiquitin during hypo- and hyper-osmotic stress in abdominal muscle and hepatopancreas (a digestive tissue) at 30, 60, and 120 min of osmotic stress. Hypo- and hyper-osmotic stress significantly increased the levels of the mRNAs encoding HSP70 and HSP90 in abdominal muscle. Hyperosmotic stress increased HSP90 mRNA levels in hepatopancreas, but hypo-osmotic stress did not. Both abdominal muscle and hepatopancreas exhibited significant changes in polyubiquitin gene expression during osmotic stress. In abdominal muscle, polyubiquitin mRNA levels increased during both hypo- and hyper-osmotic stress. Hepatopancreas, however, showed a significant elevation in polyubiquitin mRNA only during hypo-osmotic stress.

Introduction

The lobster life cycle includes several stages that experience osmotic stress. First, as pelagic larvae suspended at the surface of the water column, near-shore larval lobsters encounter hypo-osmotic stress during periods of rainfall (Ennis, 1995). As larvae (zoeal stages I–III), lobsters can tolerate reduced salinities. This tolerance, however, decreases as development proceeds to stages IV and V (Char-

mantier *et al.*, 1988). Following metamorphosis and settlement, intertidal juvenile lobsters can experience osmotic shifts due to springtime freshwater run-off, and these events can lead to mortality (reviewed in Charmantier *et al.*, 2001). Migrating seasonally into coves and estuaries to reproduce, adult lobsters may endure osmotic stress in brackish waters (Lawton and Lavalli, 1995). In contrast to pelagic larval life stages, juvenile and adult lobsters (primarily benthic organisms) are described as stenohaline and as poor osmoregulators (Dall, 1970), although they can survive in the laboratory at salinities as low as 8 ppt (McLeese, 1956) and may be exposed to salinities as low as 0 ppt during winter snow run-off (reviewed in Charmantier *et al.*, 2001).

Early studies of lobster environmental physiology were concerned with the determination of optimal and suboptimal conditions for maintaining lobsters in captivity. McLeese (1956) found that the survival of adult *Homarus americanus* was negatively impacted by various levels of hypo-osmotic stress. By acclimating lobsters over several reduced salinities, he determined that there were interactive effects of low salinity and temperature on survival (McLeese, 1956). Gonzalez and Bradley (1994) observed both up- and down-regulation in the synthesis of specific proteins in the copepod *Eurytemora affinis* in response to stress from low and high salinities.

To examine the osmotic stress response at the level of gene transcription, we used several homologous molecular probes now available for stress-responsive genes in *H. americanus* (Shean and Mykles, 1995; Chang *et al.*, 1999; Spees *et al.*, 2002). As indexes of lobster stress *in vivo*, we examined the levels of the mRNAs encoding the molecular chaperones HSP70 (= 70-kDa heat-shock protein) and HSP90 (=90-kDa heat-shock protein), and polyubiquitin. HSPs are well-characterized and pervasive "chaperones" of nascent peptides during protein synthesis; they also help to re-fold denatured proteins resulting from environmental

perturbation of cells (reviewed in Morimoto *et al.*, 1990; Hartl, 1996; Nover and Scharf, 1997; Bukau and Horwich, 1998; Feder and Hofmann, 1999). HSP90 is one of the most abundant proteins in eukaryotic cells, accounting for as much as 1%–2% of total cytosolic protein even in the absence of stress (Welch and Feramisco, 1982; Parsell and Lindquist, 1993; Jakob and Buchner, 1994).

Although HSP70 appears to be a "chaperoning generalist" assisting in the folding and re-folding of most proteins, HSP90 chaperones specific targets, many of which require its presence for final maturation (Nathan *et al.*, 1997). Classical examples of HSP90 substrates include a variety of steroid hormone receptors, the aryl hydrocarbon receptor, kinases, the heat-shock transcription factor, and the tumor-suppressor protein p53 (Buchner, 1999). HSP90 plays dual roles in environmental stress responses (Buchner, 1999; Rutherford and Lindquist, 1999) and cellular signal transduction (Pratt, 1997, 1998). HSP90 is likely to regulate the crustacean ecdysteroid (steroid molting hormone) receptor as demonstrated in *Drosophila* (Arbeitman and Hogness, 2000). In the absence of environmental stress, lobster HSP90 mRNA may be induced by injections of ecdysteroid (20-hydroxyecdysone; Chang *et al.*, 1999) and increases significantly in claw muscle during premolt atrophy (an ecdysteroid-dependent process) (Spees *et al.*, unpubl.).

During cell stress, damaged proteins that cannot be salvaged by chaperones are no longer functional and are potentially cytotoxic (Feder, 1999). The process of destroying proteins and the recycling of useful peptides and amino acids occurs *via* the ubiquitin/proteasome pathway. Ubiquitin (a 76 amino acid peptide) is bound to targets through a multi-step enzyme cascade. The resulting pattern of moieties signals a degradation complex (26S proteasome) to remove targeted proteins or regulatory subunits from the cell (Varshavsky, 1997). To expedite the production of ubiquitin, eukaryotic genetic coding regions for multiple ubiquitin molecules are linked and transcribed as a single "polyubiquitin" transcript. The resulting polypeptide is post-translationally cleaved into functional ubiquitin units. Because its rapid and robust production is correlated with environmental stress, ubiquitin (ubiquitous in cells) was identified early as one of the first "stress proteins." In mussels (*Mytilus trossulus*), conjugation of ubiquitin has been used to quantify irreversible protein damage in nature (Hofmann and Somero, 1995). Polyubiquitin mRNA levels increase severalfold in both *Manduca sexta* (Myer and Schwartz, 1996) and *Drosophila* (Lee *et al.*, 1988) following thermal stress. Quantification of polyubiquitin mRNA has also been used to examine the degradation process during premolt claw atrophy in the land crab *Gecarcinus lateralis* (Shean and Mykles, 1995). Recently, increases in ubiquitin concentrations have been observed in grass shrimp following their exposure to heat shock, cadmium, and petroleum fuels (Downs *et al.*, 2001).

Osmotic conditions strongly affect the survival of *H. americanus* (McLeese, 1956). In response to acute osmotic stress, we hypothesized that stenohaline juvenile lobsters would upregulate mRNA levels of HSP70, HSP90, and polyubiquitin—stress-responsive genes often induced during protein synthesis or turnover.

Materials and Methods

Animal care

Lobsters were reared from larvae hatched in a flow-through aquaculture system at the Bodega Marine Laboratory, Bodega Bay, California. Detailed descriptions of our larval-rearing and aquaculture system are available elsewhere (Chang and Conklin, 1993; Conklin and Chang, 1993). Intermolt juvenile male lobsters were used for all experiments. Animals were molt-staged based upon microscopic examination of pleopod cuticular development (Aiken, 1973). Prior to the osmotic stress experiments, the salinity of the flow-through system was stable (32 ppt) for several weeks.

Osmotic stress

Two-liter jars were fitted with air stones and filled with either 1.0 l of 100% seawater (controls; 32 ppt), 50% seawater (0.5 l seawater with 0.5 l distilled and 0.2 μm-filtered freshwater; 16 ppt), or 150% seawater (1.0 l total volume with 16 g additional dissolved sea salts; 48 ppt; Sigma). Salinity was determined with a refractometer. The jars were thermally equilibrated by partial submersion in the flow-through system at 15 °C, the rearing temperature of the juvenile lobsters. Lobsters ($n = 4$ for all treatments) were removed from their compartments and placed directly into the jars, where they remained for 30, 60, or 120 min. During these time points we have previously measured significantly elevated levels of HSP70 and HSP90 mRNAs in lobsters exposed to acute thermal stress (Spees *et al.*, 2002). Controls (100% seawater) were incubated for 30, 60, and 120 min in the same manner. Following all of the treatments (100% survival), animals were quickly removed from the jars and sacrificed. Samples of abdominal muscle and hepatopancreas (midgut gland) were dissected, frozen in liquid N_2, and stored at -70 °C.

We ran a parallel experiment to examine the effect of salinity on hemolymph osmolarity. Hemolymph (50 μl) was removed repeatedly from individual lobsters at 0, 30, 60, and 120 min of exposure to 50, 100, or 150% seawater ($n = 4$ for each time point), and osmolarity was measured with a vapor pressure osmometer (Wescor). The osmolarity of seawater from the flow-through system was 940 mos$M$. Student's t test was used to compare the hemolymph osmolarity of animals incubated in 100% seawater to that of animals incubated in 50% or 150% seawater.

Northern analysis

Total RNA was isolated from the abdominal muscle and hepatopancreas samples (RNAgents kit, Promega), quantified by absorbance at 260 nm with a spectrophotometer, and equally loaded (15 μg abdominal muscle total RNA; 25 μg hepatopancreas total RNA) onto denaturing (formaldehyde) 1% agarose gels. These gels were washed (15 min, diethylpyrocarbonate water); blotted overnight onto nylon membranes (Magnagraph, MSI), UV cross-linked; and prehybridized (2 h) in 5× SSPE buffer (0.75 M NaCl, 50 mM NaH$_2$PO$_4$, 5 mM ethylenediaminetetraacetic acid, pH 7.4), 50% (w/v) formamide, 5× Denhardt's, 1% sodium dodecyl sulfate (SDS), and 100 μg/ml denatured sheared salmon sperm DNA at 42 °C. A partial lobster HSP90 clone (350 bp, cloning described in Chang et al., 1999) was ^{32}P-labeled (Prime-It RmT, Stratagene), added directly to the prehybridization solution, and allowed to hybridize overnight at 42 °C. Following hybridization, the blots were washed twice with 2× SSPE at room temperature and placed on film overnight at −70 °C.

Following exposure of the film, the blots were stripped with several washes of buffer (15 mM NaCl, 1.5 mM sodium citrate, pH 7, with 0.1% SDS at 65 °C) until background was minimal; they were then prehybridized, hybridized, and washed as above, except that a partial lobster polyubiquitin cDNA probe was added (690 bp fragment from Acc I digest of lobster polyubiquitin clone; described in Shean and Mykles, 1995). To check for equal loading of RNA, the blots were probed with a partial lobster actin cDNA (700 bp, Harrison and El Haj, 1994). Lastly, following similar washes and prehybridization, a partial lobster HSP70 cDNA probe was hybridized with the blots (500 bp, Spees et al., 2002). Films were scanned on a high-resolution scanner, and densitometry was performed with NIH Image software. The signals from these northern blots were quantified, normalized against the actin signal to control for equal loading of RNA, and expressed as percent control mRNA level. All data were log$_{10}$-transformed to normalize variance prior to statistical analysis. Student's t test was used to identify differences between treatment and control mRNA levels. P values ≤0.01 were considered significant for the mRNA experiments and ≤0.05 for the hemolymph osmolarity measurements.

Results

Hemolymph osmolarity during acute osmotic stress

Hemolymph osmolarity differed significantly between control animals and those incubated in either hypo- or hyper-osmotic conditions at all time points examined after the start of the experiments (Fig. 1). As expected, the hemolymph osmolarity of animals incubated in 50% seawater decreased over time (30 min, $P < 0.001$; 60 min,
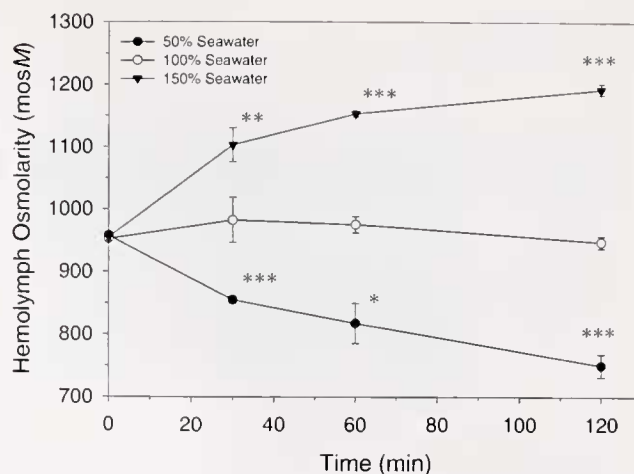

Figure 1. Hemolymph osmolarity (mos$M$) of individual lobsters repeatedly sampled at 0, 30, 60, and 120 min of exposure to control (100%), hypo-osmotic (50%), or hyper-osmotic (150%) seawater. For each time point, $n = 4$; error bars represent one standard deviation of the mean. Asterisks indicate significant difference between treatment and respective control at a given exposure time (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

$P = 0.029$; 120 min, $P < 0.001$; Fig. 1). In contrast, the hemolymph osmolarity of animals incubated in 150% seawater increased over time (30 min, $P = 0.002$; 60 min, $P < 0.001$; 120 min, $P < 0.001$; Fig. 1).

Gene expression in response to osmotic stress

Representative northern blots from analysis of total RNA are shown for lobster abdominal muscle dissected from animals that received hypo- and hyper-osmotic stress for 30, 60, and 120 min (Fig. 2A-C). We had previously determined that transfer of lobsters from their rearing system into 2-l jars did not in itself alter HSP transcription levels (Spees et al., 2002).

HSP70 mRNA levels

In abdominal muscle, HSP70 mRNA levels were significantly increased by both hypo- and hyper-osmotic stress (Fig. 3). HSP70 mRNA levels were significantly higher than control levels by 30 min of incubation in 50% seawater ($P = 0.002$) and continued to be elevated at 60 min of incubation ($P = 0.002$). Exposure to 150% seawater resulted in a significant elevation of abdominal muscle HSP70 mRNA levels over control levels at 60 min ($P = 0.004$). HSP70 mRNA levels returned to control levels in both salinity exposure groups by 120 min. HSP70 expression was not measured in hepatopancreas.
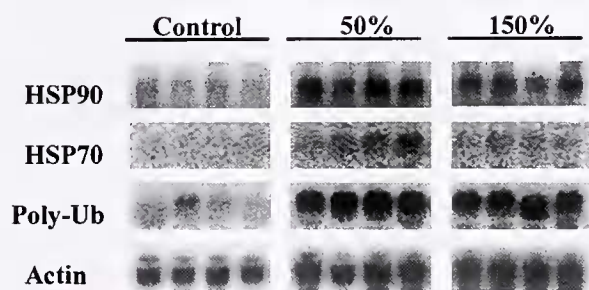
HSP90 mRNA levels

Both hypo- and hyper-osmotic stress significantly increased HSP90 mRNA levels in lobster abdominal muscle

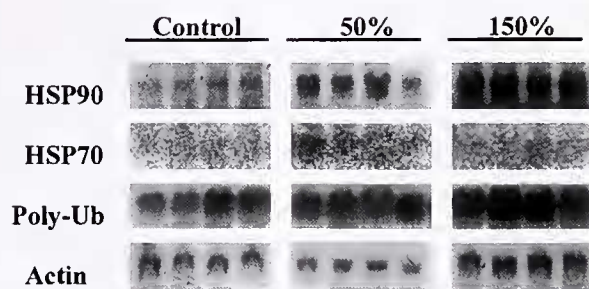## A) 30 min
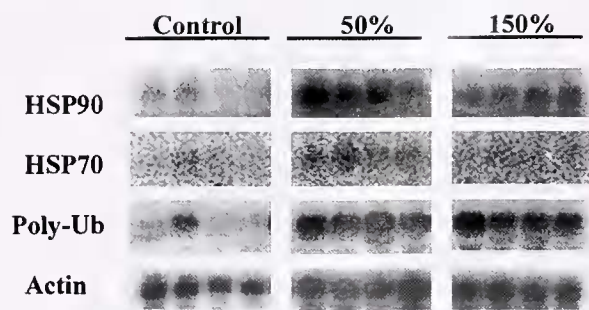
B) 60 min

C) 120 min

Figure 2. Northern analysis of total RNA showing HSP70, HSP90, and polyubiquitin gene expression in response to control (100% seawater), hypo-osmotic (50% seawater), or hyper-osmotic (150% seawater) stress in *Homarus americanus* abdominal muscle at (A) 30, (B) 60, and (C) 120 min of exposure. Northern blots were serially probed with ^{32}P-labeled partial cDNAs for lobster HSP70, HSP90, polyubiquitin, and actin (indicator of equal loading). At each salinity and for each probe, RNA from a single lobster was run in each of the four lanes.

Figure 3. Quantitative analysis of lobster HSP70 gene expression in abdominal muscle during hypo- and hyper-osmotic stress (50% and 150% seawater) for 30, 60, and 120 min. Data are normalized against the actin signal (indicator of equal loading) and expressed as percent control (100% seawater) mRNA level. For all time points, $n = 4$; error bars represent one standard deviation of the mean. Significance between treatment and control HSP70 mRNA levels is indicated ($**P \leq 0.01$).

cle HSP90 mRNA levels were significantly increased over control levels by 30 min of exposure ($P = 0.001$) and remained elevated at the 60-min ($P = 0.009$) and 120-min ($P = 0.01$) time points.

Hepatopancreas responded differently to the hypo-osmotic treatment. In 50% seawater, HSP90 mRNA levels never rose significantly above control levels. Hepatopancreas HSP90 gene expression did, however, change in re-

Figure 4. Quantitative analysis of lobster HSP90 gene expression during hypo- and hyper-osmotic stress (50% and 150% seawater) for 30, 60, and 120 min in abdominal muscle. Data are normalized against the actin signal (indicator of equal loading) and expressed as percent control (100% seawater) mRNA level. For all time points, $n = 4$; error bars represent one standard deviation of the mean. Significance between treatment and control HSP90 mRNA levels is indicated ($**P \leq 0.01$, $***P \leq 0.001$).

at all time points examined (Fig. 4). In 50% seawater, HSP90 mRNA levels were significantly greater than control levels by 30 min of exposure ($P < 0.001$). HSP90 mRNA levels also remained significantly elevated at the 60-min ($P < 0.001$) and 120-min ($P = 0.008$) time points of the hypo-osmotic treatment. In 150% seawater, abdominal mus-
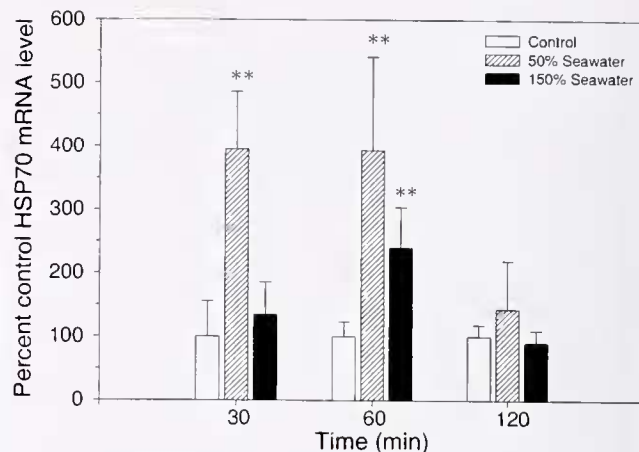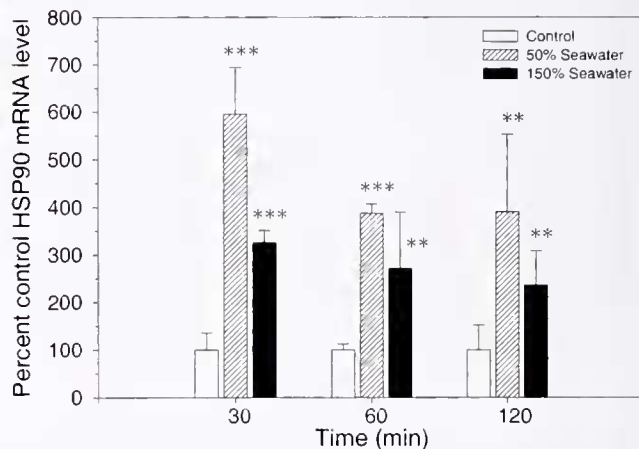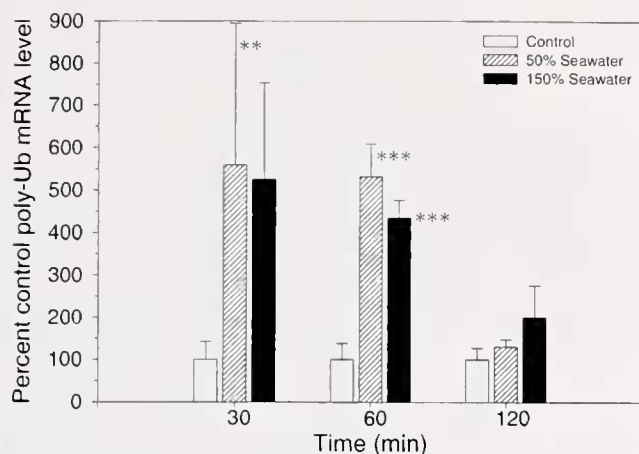
Figure 5. Quantitative analysis of lobster polyubiquitin gene expression during hypo- and hyper-osmotic stress (50% and 150% seawater) for 30, 60, and 120 min in abdominal muscle. Data are normalized against the actin signal (indicator of equal loading) and expressed as percent control (100% seawater) mRNA level. For all time points, $n = 4$; error bars represent one standard deviation of the mean. Significance between treatment and control HSP90 mRNA levels is indicated (**$P \leq 0.01$, ***$P \leq 0.001$).

sponse to the hyper-osmotic treatment. In 150% seawater, HSP90 mRNA levels were significantly different than control levels at the 120-min time point ($P = 0.008$; mean $\pm$ standard deviation $= 438\% \pm 205\%$ of control values).

Polyubiquitin mRNA levels

Hypo- and hyper-osmotic stress both significantly increased polyubiquitin mRNA levels in abdominal muscle (Fig. 5). Significant elevation over control levels was detectable by 30 min of exposure in 50% seawater ($P = 0.004$). Polyubiquitin mRNA levels were significantly elevated at 60 min of exposure to either salinity treatment (50% seawater, $P < 0.001$; 150% seawater, $P < 0.001$).

Significant elevation of hepatopancreas polyubiquitin mRNA was detected at 60 min of exposure to 50% seawater ($P = 0.007$; 227% $\pm$ 50.5% of control values). Hyperosmotic stress did not lead to significant increases in hepatopancreas mRNA levels for any of the exposures we tested.

Discussion

Our results demonstrate that, for *Homarus americanus,* (1) exposure to 50% or 150% seawater significantly alters hemolymph osmolarity, (2) hypo- and hyper-osmotic stress both can significantly elevate molecular chaperone gene expression, (3) osmotic effects on molecular chaperone mRNA levels may differ depending on duration of stress and tissue, and (4) polyubiquitin gene expression is also significantly increased by osmotic stress and similarly can differ on the basis of exposure duration and tissue.

Tissue-specific patterns of stress-responsive gene expression

We observed tissue-specific patterns of lobster HSP90 expression following osmotic challenge. Whereas HSP90 expression was significantly increased in lobster abdominal muscle by hypo-osmotic treatments, expression in hepatopancreas was unaffected by any duration of hypo-osmotic stress that we tested. Both tissues, however, displayed significant elevations in HSP90 mRNA levels in response to hyper-osmotic stress. As the composite protein pools of abdominal muscle and hepatopancreas may differ considerably, variation in expression patterns may be due to differential stability of proteins arising from ionic or other solute interactions.

In lobster abdominal muscle and hepatopancreas, HSP70 and HSP90 mRNA levels are both raised by thermal stress (Chang *et al.,* 1999; Spees *et al.,* 2002) and by osmotic stress in this study (only HSP90 mRNA was examined in hepatopancreas). Abdominal muscle appears to be a more thermally stable tissue than hepatopancreas (*in vivo* expression of polyubiquitin does not occur in abdominal muscle from the same lobsters that do express it in the hepatopancreas during and after a 13 °C acute heat-shock; Spees *et al.,* 2002). In the present study, however, abdominal muscle did show an increase in expression of polyubiquitin during both hypo- and hyper-osmotic stress. In fact, it did so to a much greater extent than hepatopancreas, which did not show a significant increase in polyubiquitin mRNA levels in response to any duration of hyper-osmotic stress that we tested. Although less sensitive to thermal stress, abdominal muscle appears to be more sensitive than hepatopancreas to osmotic stress.

Does osmotic stress cause protein instability or denaturation in vivo?

It is thought more likely that low availability of ions perturbs cells by affecting enzyme-ligand interactions rather than by altering enzyme conformations substantially (protein structure) (Somero and Yancey, 1997). The cations Na^+, K^+, Mg^{+2}, Ca^{+2}, and the anion Cl^- are all present in seawater and are known to destabilize proteins at excessive concentrations (>1 M) (Somero and Yancey, 1997). *In vitro* results from 3T3 and SV-3T3 (rat) cells in culture show that the presence of the osmolyte betaine (*N*-trimethylglycine) reduces the production of HSP70 during hypertonic (500 mos$M$) incubation (Petronini *et al.,* 1993). Betaine also inhibits HSP70 protein induction in Madin-Darby canine kidney (MDCK) cells during thermal stress (Sheikh-Hamad *et al.,* 1994), suggesting that, as is commonly observed during thermal stress, some form of protein destabilization may occur during osmotic stress. Interestingly, the heat-shock transcription factor (HSF1) can be activated by either hyper- or hypo-osmotic stress in mammalian (HeLa) cells,

but does not induce HSP70 mRNA, indicating that it may play a different role in regulating osmo-sensing pathways or osmotic stress proteins (Caruccio *et al.*, 1997).

Because the hypo- and hyper-osmotic conditions used in this study may not generate ionic conditions *in vivo* that have been observed to denature proteins *in vitro*, it is important to consider alternative hypotheses concerning osmotic stress and stress-responsive gene expression. In the rat kidney, both the mRNA and protein levels of HSP72 and HSP25/27 increase steeply along the corticopapillary axis in a pattern that matches tissue solute levels in the distal tubule. It is believed, however, that hypertonicity rather than hyperosmolarity *per se* is actually responsible for these patterns, because increased HSP synthesis correlates with the addition of relatively membrane-impermeable substances (NaCl) and not with the addition of membrane-permeable substances (urea). Thus, alterations in membrane fluidity or shrinking and swelling may influence HSP expression in some cellular systems without protein denaturation (reviewed in Beck *et al.*, 2000).

Increased molecular chaperone mRNA levels may be required in a particular tissue for signal transduction or osmolyte responses to intracellular ionic changes. Pan *et al.* (2000) found that, in salmon, hyper-osmotic stress could raise the levels of HSP90 mRNA in the branchial lamellae (both *in vitro* and *in vivo*), but not in the kidney. Because cortisol is believed to govern osmoregulatory capacity in salmon by influencing chloride cell differentiation and ATPase activity in the gill, and HSP90 is known to regulate glucocorticoid receptors, Pan *et al.* (2000) hypothesized that HSP90 might be playing a specific role in signal transduction during osmotic stress. In the lobster, there is evidence that molting hormone (ecdysteroid) titers can influence molecular chaperone gene expression (Chang *et al.*, 1999; Spees *et al.*, unpubl.).

The changes in lobster polyubiquitin gene expression we observed during osmotic stress indicate that protein turnover is occurring. Although the osmotic stress used in this study may not be excessive enough to denature proteins that have achieved their native state, it may have been enough to affect the folding of nascent proteins post-translation. As linear molecules arising from the ribosome, proteins may be more susceptible to ionic fluctuations. Improperly folded proteins in this case would be ubiquitinated and degraded by the 26S proteasome. Alternatively, the synthesis of proteins required for osmotic stress responses could account for the molecular chaperone gene expression we observed, while degradation (ubiquitination) of proteins that are unnecessary or detrimental during osmotic stress could account for increased polyubiquitin mRNA levels. Further study will be required to determine the effects on gene expression of osmotic stresses that are not as extreme as those that we used and that are more like the stresses encountered in nature. In addition, studies will be needed to elucidate the mechanisms responsible for our observations and the extent to which ionic fluctuations can alter protein stability *in vivo*.

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